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The Contribution of Somatic Hypermutation to the Diversity of Serum Immunoglobulin: Dramatic Increase with Age

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Summary

Although somatic mutation contributes to the diversity of only a minor fraction of B cells in mouse spleen or blood, its contribution to the diversity of serum immunoglobulin is unknown. We have devised an immunoassay to monitor mutated antibodies in serum using a monoclonal antibody that recognizes a Vκ only when mutated at its major intrinsic hot spot. Mutation makes essentially no contribution to the diversity of endogenous serum IgM, IgG, or IgA in young mice. However, in response to environmental antigens, the titer of mutated immunoglobulin in T cell–proficient mice rises strikingly with age, such that the major proportion of serum immunoglobulin in adults is somatically mutated, with the mutation load in IgG being some 10-fold greater than in IgM.

Introduction

Functional immunoglobulin genes are produced by integration of germline V, D, and J gene segments during lymphocyte development, with this DNA rearrangement yielding junctional and combinatorial diversity. In a proportion of B lymphocytes, these functional immunoglobulin genes are further diversified by somatic hypermutation. The contribution of somatic mutation is routinely analyzed at the cellular level, comparing the sequences of the V genes expressed in a particular B cell population to those of their presumed germline counterparts. From such studies, it is evident that most mouse splenic B cells express unmutated V genes and that somatic mutation contributes to the diversity of only a minor fraction of the total B cell population of mouse peripheral blood, although mutation is a major contributor to V gene diversity among B cells in the germinal centers of peripheral lymphoid organs (MacLennan and Gray, 1986; Berek et al., 1991; Gu et al., 1991; Jacob et al., 1991).

However, in contrast to cell-mediated immunity, the protective arm of humoral immunity is afforded not by the lymphocytes themselves, but rather by the immunoglobulin present in secretions. Little is known about the contribution of somatic mutation to serum immunoglobulin diversity; extrapolating from the pattern of diversity within different B lymphocyte subpopulations is not trivial. We therefore sought to devise a serological assay that would enable us to assess the contribution of somatic mutation to serum immunoglobulin diversity. We were essentially following the parallel of class-switch recombination where it is serological assays that underpin our knowledge that switched isotypes account in molar terms for >90% of serum immunoglobulin but <10% of total B cells in spleen and blood.

The strategy that we have used exploits the fact that the nucleotide substitutions introduced by somatic hypermutation are not distributed randomly throughout the V gene segment but rather exhibit a favored targeting to individual hot spots (Sharpe et al., 1991; Rogozin and Kolchanov, 1992; Betz et al., 1993). In the case of mouse V_κOx1, some 16% of mutated sequences carry nucleotide substitutions at a Ser-31 hot spot in CDR1 (Figure 1A), the majority of which change this AGC codon to AAC, yielding an asparagine. Our strategy was, therefore, to isolate a monoclonal antibody (mAb) that recognizes the Asn-31 (but not Ser-31) version of V_κOx1 and use this mAb to monitor the abundance of mutated immunoglobulin in mouse sera.

Results

Distinguishing Mutated V_κOx1 Using JEN31 mAb

To isolate an antibody specific for the mutated form of V_κOx1 that carries a Ser→Asn substitution at the codon 31 hot spot (V_κOx-N^{31}), mice were immunized with thyroglobulin to which had been coupled a 13-mer peptide whose sequence corresponded to the CDR1 of V_κOx-N^{31}. Hybridomas were established from eight hyperimmunized mice and the supernatants screened by ELISA for the presence of peptide-specific antibody.

In order to ascertain whether any of these mAbs could recognize V_κOx-N^{31} and distinguish it from the parental V_κOx-S^{31}, DNA transfection into NS0 myeloma cells was used to prepare a matched pair of recombinant anti-2-phenyl-oxazolone IgM antibodies (V_HOx1/V_κOx1-N^{31} and V_HOx1/V_κOx1-S^{31}). A single hybridoma (designated JEN31) was identified that showed a strong discriminatory ability and was cloned by limiting dilution (Figure 1B). Recognition by JEN31 did not depend on the nature of the V_H with which V_κOx-N^{31} was paired; good binding was obtained to a recombinant V_HMOPC21/V_κOx-N^{31} Fab fragment (Figure 1C) as well as to isolated light chains (see below).

ELISAs revealed that the JEN31 mAb (which is an IgGκ) recognized the recombinant V_HOx1/V_κOx1-N^{31} IgM antibody (as well as the corresponding Fab fragment) when the IgM was presented directly bound to the plastic plate but not if it was indirectly captured on the plate by an antigen or anti-IgM tether (Figures 1B and 1C). Thus, JEN31 does not appear to recognize the V_κOx1-N^{31} CDR1 when encountered in its native configuration. The JEN31 mAb can, however, be used to detect V_κOx1-N^{31} light chains in a Western blot assay and discriminate them from V_κOx1-S^{31} light chains (Figure 1D). In such an assay, JEN31 can detect down to around 1 ng of V_κOx1-N^{31} light chain, whereas not even a faint signal is obtained with as much as 10 μg of V_κOx1-S^{31} κ chain.

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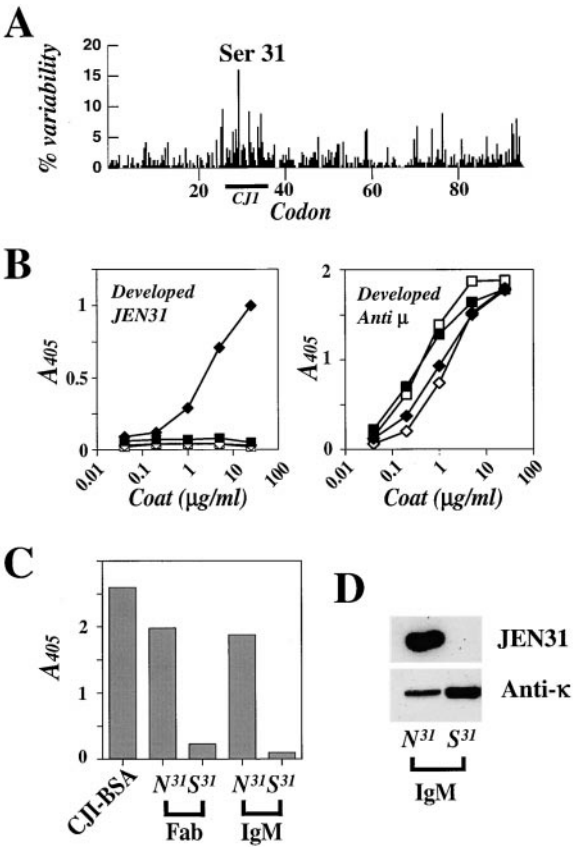

Figure 1. Isolation and Characterization of mAb JEN31

(A) The distribution of unselected mutations in VκOx1, indicating the CDR1 peptide used for the immunization. The distribution (kindly provided by Cristina Rada) is based on a compilation of mutations identified in this lab in V_κOx1 transgenes from Peyer's patch germinal center B cells. Note that although 16% of mutated sequences carry a mutation at Ser-31, in only 9% of the sequences does the mutation yield a Ser→Asn substitution.

(B) JEN31 discriminates V_κOx1-N^{31} from V_κOx1-S^{31} when directly coated onto plastic plates, as determined by ELISA. Various concentrations of either [V_HOx1/V_κOx1-N^{31}] (rhombi) or [V_HOx1/V_κOx1-S^{31}] (squares) anti-2-phenyloxazolone recombinant IgM antibodies were either directly coated onto plastic plates (filled symbols) or tethered onto plates that had been coated with phOx-BSA (open symbols). The plates were then incubated with either biotinylated JEN31 (left-hand panel) or biotinylated goat anti-mouse μ and developed with avidin-conjugated HRP.

(C) JEN31 distinguishes [V_HOx1/V_κOx1-N^{31}] from [V_HOx1/V_κOx1-S^{31}] Fab fragments. The binding of biotinylated JEN31 to wells of a plate that had been directly coated with saturating concentrations of the indicated IgM, Fab fragment, or CJ1-BSA conjugate was monitored by ELISA.

(D) JEN31 discriminates V_κOx1-N^{31} (1 μg loaded) from V_κOx1-S^{31} (3 μg loaded) in a Western blot assay. Sample loading was confirmed in a parallel blot probed with anti-rat κ.

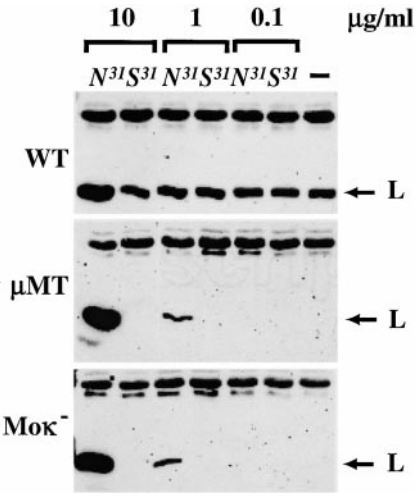

Figure 2. JEN31 mAb Discriminates V_κOx1-N^{31} from V_κOx1-S^{31} Igκ Chains in the Context of Total Mouse Serum

Recombinant [V_HOx1/V_κOx1-N^{31}] and [V_HOx1/V_κOx1-S^{31}] IgMs were dosed into serum from adult normal, μ*MT* (B cell and immunoglobulin-deficient), or *Moκ*$^{-/-}$ (normal serum immunoglobulin levels but with IgL chain solely of λ isotype) mice at the concentrations indicated and samples (10 μl) of the dosed serum subjected to SDS/PAGE and Western blotting with JEN31. "L" marks the migration position of IgL chains.

Assaying the Abundance of Mutated Ig Using JEN31

To ascertain whether JEN31 could discriminate V_κOx1-N^{31} from V_κOx1-S^{31} Igκ chains in the context of normal mouse serum, the two recombinant IgM anti-phenyloxazolone antibodies were dosed into mouse serum and analyzed by Western blotting. Although it is possible to distinguish the two types of IgM-dosed sera when the dosing is at 10 μg/ml, the sensitivity of the detection is limited by a high background; normal mouse serum already contains JEN31-reactive material that migrates with the transgenic Igκ chain (Figure 2). This background is due to a product of the endogenous mouse Igκ locus, since it is absent from the serum of both μ*MT* mice (which contain no serum immunoglobulin) and *Moκ*$^{-/-}$ mice (which carry a targeted disruption of Cκ but harbor normal amounts of serum immunoglobulin but with the IgL chains being solely of the λ isotype [Zou et al., 1995]).

These results suggested that it might be possible to follow the somatic mutation of a V_κOx1-containing Igκ transgene by Western blot analysis of total serum, particularly if the endogenous light chains were only of the λ isotype. *L*κ mice (which carry an Igκ transgene that is composed of an unmutated mouse V_κOx1/J_κ5 rearrangement linked to rat Cκ) were serially crossed with *Moκ*$^{-/-}$ mice and Western blot analysis performed using sera derived from *Lκ*$^+$*[Moκ*$^{-/-}$*]* progeny that had been born to *Lκ*$^-$*[Moκ*$^{-/-}$*]* mothers, thereby avoiding complications arising due to maternally transmitted immunoglobulin.

The serum from unimmunized *Lκ*$^+$*[Moκ*$^{-/-}$*]* mice but not from control *Lκ*$^-$*[Moκ*$^{-/-}$*]* mice yielded a clear signal with the JEN31 antibody (Figure 3A). Since we had been unable to detect any binding of JEN31 to unmutated Lκ, we assumed that immunoglobulin containing somatically mutated Lk light chains must be present in the serum of the *Lκ*$^+$*[Moκ*$^{-/-}$*]* mice and that this is generated even in the absence of intentional immunization.

We have previously described mice carrying a transgene (LκΔ[$Int_{J\text{-}C}$, 3′Fl]) that is derived from Lκ but that harbors intronic deletions such that, while well expressed, it constitutes a poor substrate for the somatic hypermutation mechanism (Klix et al. 1998; Figures 3B and 3C). If the JEN31-reactive material in the sera of *Lκ*$^+$*[Moκ*$^{-/-}$*]* mice is indeed mutated transgenic κ chain, then one would expect *LκΔ[*$Int_{J\text{-}C}$*, 3′Fl]*$^+$ *[Moκ*$^{-/-}$*]* mice

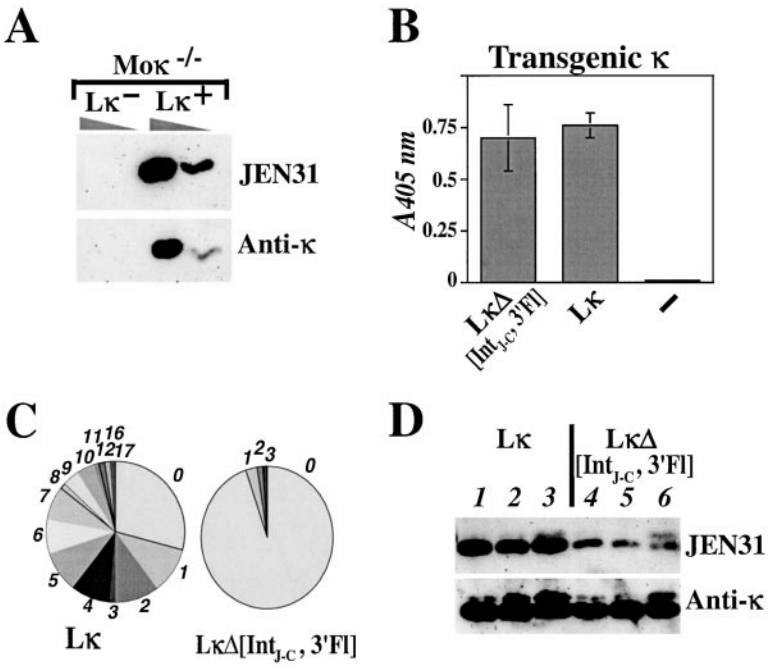

Figure 3. Differential Accumulation of Mutated V_κOx1 Antibodies in Mice Carrying Lκ and LκΔ[$Int_{J\text{-}C}$, 3′Fl] Transgenes

(A) Comparison of JEN31-reactive material in sera (0.5 and 0.05 μl samples) from 6-month-old *Lκ$^+$[Moκ$^{-/-}$]* and *[Moκ$^{-/-}$]* mice; blots were reprobed for Igκ.

(B) Comparison of the concentration of transgenic κ-containing total immunoglobulin in sera of 6-month-old Lκ and LκΔ[$Int_{J\text{-}C}$, 3′Fl] transgenic mice; six mice were titered per group with the ELISA results presented performed using a serum dilution (50-fold) in which the assay is within the range of proportionality.

(C) Comparison of transgene hypermutation in *Lκ* and *LκΔ[$Int_{J\text{-}C}$, 3′Fl]* mice. The data (taken from Klix et al., 1998) are presented as pie charts and indicate the proportions of transgene sequences PCR amplified from Peyer's patch germinal center B cells (out of a total of about 75) that contain 0, 1, 2, etc., mutations in the V domain.

(D) Monitoring the accumulation of mutated transgenic Igk chain by JEN31 Western blot using sera from three 10-week-old *LκΔ[$Int_{J\text{-}C}$, 3′Fl]$^+$ [Moκ$^{-/-}$]* and three age-matched *Lκ$^+$ [Moκ$^{-/-}$]* mice. A parallel blot was probed with an anti-κ mAb to control for loading.

to give a much weaker JEN31 signal. This is indeed the case (Figure 3D).

Age-Dependent Increase in Abundance of Mutated Immunoglobulin

Thus, somatically mutated antibody is already present in the serum of adult mice that have been housed in a barriered, specific pathogen free facility—without intentional immunization.

The time course of the appearance of such mutated antibody was monitored by JEN31 Western blot analysis of serum samples obtained from *Lκ$^+$[Moκ$^{-/-}$]* mice that had been born to *Lκ$^-$[Moκ$^{-/-}$]* mothers. Somatically mutated antibody is hardly detectable before weaning (Figure 4A), but a very weak JEN31 signal is obtained at 5 weeks (Figure 4B). A time course analysis revealed that the titer of JEN31-reactive material increased steadily with age, and a cohort of a dozen mice was followed for 4 months to compare the increase in abundance of endogenously synthesized mutated immunoglobulin with that of class-switched isotypes (Figure 4D).

It will be seen that IgM levels are already high at 10 days of age (around 50% of adult levels), although they increase further over the next few weeks. In contrast, IgG and IgA titers largely develop after weaning, plateauing at around 7 weeks. Mutated immunoglobulin only becomes readily detectable at 6–7 weeks (when serum IgA titers have effectively plateaued) but then goes on to increase consistently and steadily over months. Indeed, although the main cohort of mice was only followed for 4 months, analysis of three members of the cohort reveals that the abundance of mutated immunoglobulin continues to increase even beyond 6 months (Figure 4C), while total IgM, IgG, and IgA titers change little. Although there is inevitably some variation between animals, the tendency is remarkably consistent.

The Extent of the Contribution of Somatic Mutation

Thus, there appears to be a dramatic shift in the repertoire of serum immunoglobulin that takes place long after the concentrations of serum IgM, G, and A have plateaued. To estimate the extent to which mutated immunoglobulin contributes to the antibody repertoire of older mice, we quantified the intensity of the JEN31 reactivity of the Igκ chains in sera of 1-year-old mice, using the Igκ chains of purified [V_HOx1/V_κOx1-N^{31}] IgM for calibration (Figure 5A). Densitometry of the Western blot, performed using dilutions within the range of linearity, indicated that about 6% of Lκ chains in 1-year-old mice *Lκ$^-$[Moκ$^{-/-}$]* carried a Ser-31→Asn mutation (Figure 5A). Since previous analyses of intrinsic mutational targeting indicate that about 9% of all mutated VκOx1 genes carry a Ser-31→Asn substitution (see legend to Figure 1A), these results indicate that around two-thirds of the serum immunoglobulin in a 1-year-old mouse is mutated. Clearly, there is a significant degree of uncertainty in these calculations. Not all Lκ chains carrying a Ser-31→Asn mutation will be equally reactive with JEN31; linked mutations could affect the binding affinity. Similarly, there is a degree of uncertainty in extrapolating from the proportion of Lκ chains that are JEN31$^+$ to the proportion of Lκ chains that carry any somatic mutations. Nevertheless, the results strongly indicate that a major proportion of serum immunoglobulin in an old animal is mutated; the mutation load is around two orders of magnitude higher than in a 7-week-old mouse, although mice of the two ages show little difference in serum IgG concentration.

When asserting that the serum immunoglobulin in young mice is essentially unmutated, the analysis refers to endogenously synthesized immunoglobulin. Freshly weaned mice are still, however, afforded protection by mutated immunoglobulin that originates from maternal transfer (Figure 5B).

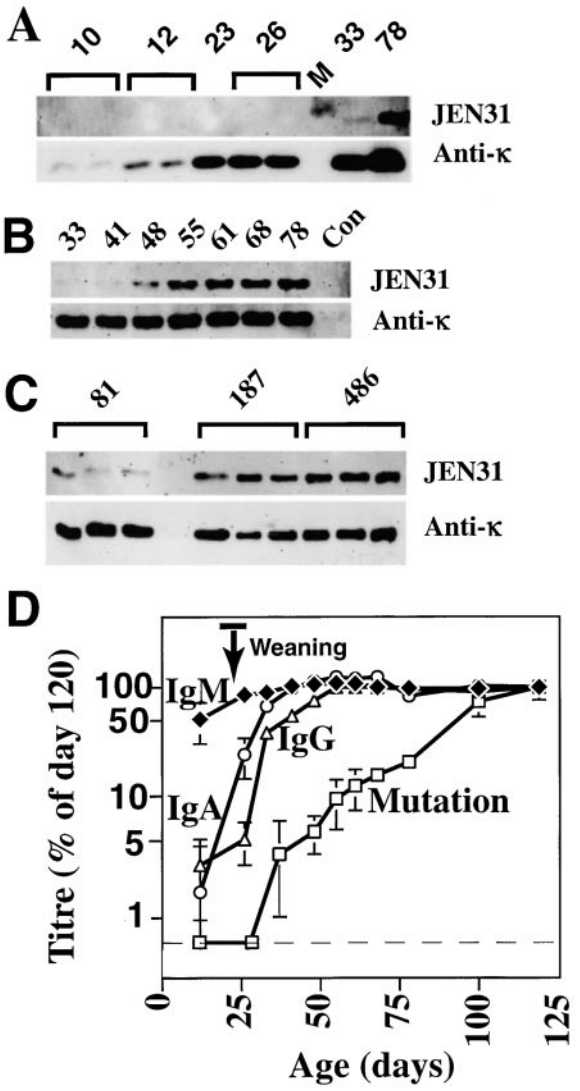

Figure 4. Class-Switched and Mutated Immunoglobulin Accumulate with Age but with Very Different Kinetics

The titer of endogenously synthesized mutated serum immunoglobulin was assessed by JEN31 Western blot analysis of sera of $L\kappa^+$ *Mo*[$\kappa^{-/-}$] mice born to $L\kappa^-$ *Mo*[$\kappa^{-/-}$] mothers.

(A) Data from preweaned mice (days 10, 12, 23, and 26) are each from sera (0.2 μl) from individual animals, whereas the day 33 and 78 samples are from the same mouse that is analyzed in (B). The blot was reprobed with anti-κ to control for loading. M, marker lane (30,000 molecular weight marker visible); Con, serum from an $L\kappa^-$ *Mo*[$\kappa^{-/-}$] negative control.

(B) JEN31 immunoblot analysis of samples (0.2 μl) taken from days 33 to 78 from an individual member of the cohort presented in (D).

(C) JEN31 immunoblot analysis of sera taken from three older $L\kappa^+$ *Mo*[$\kappa^{-/-}$] mice at the ages (days) indicated. The ratio of IgM, IgG, and IgA titers at days 486 and 187 relative to those at day 81 were IgM (1.3 and 1.3), IgG (1.1 and 1.1), and IgA (0.9 and 1.1). Loadings for individual serum samples (in the range 0.08 to 0.30 μl) were adjusted so that signals were within the range of linearity.

(D) Quantification of the mean titers of endogenously synthesized (as opposed to maternally derived) IgM, IgG, IgA, and mutated (JEN31-reactive) serum immunoglobulin presented as a function of age. All titers are given as a percentage of the day 120 titer. For the postweaning titers, the data derive from following a cohort of 12 $L\kappa^+$ *Mo*[$\kappa^{-/-}$] mice born to $L\kappa^-$ *Mo*[$\kappa^{-/-}$] mothers; the prewean titers come from a separate pool of individual animals. IgM/G/A titers were determined by ELISA, developing with anti-rat κ, so as to restrict the analysis to endogenously synthesized immunoglobulin. The titer of JEN31-reactive material was determined by Western blot, with samples diluted so as to be in the linearity range.

In Adults, Switched Isotypes Are More Mutated Than IgM

The relative contribution of somatic mutation to the diversity of serum IgM, as opposed to class-switched isotypes, is not known. Although most splenic IgM^+IgD^{hi} and peritoneal B cells in the mouse express unmutated V genes (Gu et al., 1991), it has become evident that a significant fraction of the IgM^+ B cells in human peripheral blood express mutated V genes (Huang et al., 1992; van Es et al., 1992; Klein et al., 1997). We therefore fractionated the sera of 1-year-old $L\kappa^+$[$\kappa^{-/-}$] mice by gel filtration, generating a high molecular weight fraction (in which the immunoglobulin was practically solely of the IgM isotype) and a low molecular weight fraction (predominantly composed of IgG and IgA). Analysis of the JEN31 reactivity of the various samples reveals that mutated antibodies exist in both IgM and IgG/A fractions. However, the mutation load in the switched isotype fraction is about 10-fold higher than in the IgM fraction (Figure 5C).

These results strongly indicate that, even if the analysis is restricted to serum IgG, the mutation load in old mice must be much higher than in young animals. This is indeed the case (Figure 5D).

Diminished Titers of Mutated Ig in Germ-Free Mice

The development of mutated immunoglobulin in the sera of specific pathogen free mice does not reflect an immune response to any obvious infection. A remarkably similar time course of development of JEN31-reactive antibody was observed in mice housed in different parts of the barriered animal facility (the stock breeding unit, the transgenic breeding unit, or in the isolators for maintenance of immunodeficient mice) and occurred in animals that revealed no pathology at autopsy. The development of mutated immunoglobulin with age could be most readily attributed to the challenge provided by environmental antigens such as commensal organisms in the gut. We therefore wished to monitor the abundance of mutated immunoglobulin in the serum of germ-free mice.

Although we had only used JEN31 mAb to detect mutated forms of the Lκ transgene, we wondered (since we did not have germ-free $L\kappa^+$[*Mo*$\kappa^{-/-}$] available) whether JEN31 could also be used to detect mutated forms of endogenous Igκ chains. That the Igκ locus of normal mice can indeed give rise to JEN31-reactive light chains is indicated by Western blot comparison of serum from normal and *Mo*$\kappa^{-/-}$ mice (Figure 2). It is likely that this JEN31-reactive material is due to somatically mutated Igκ chains. First, the Igκ locus in BALB/c mice has been characterized in detail (Thiebe et al., 1999) and V_κOx1-related genes analyzed in several inbred mouse strains (Even et al., 1985; Kaartinen and Mäkelä, 1987; Kaartinen et al., 1989). These studies reveal only a single V_κ with a CDR1 related to that of V_κOx1 but without a serine codon at position 31. This V_κ gene (*R13*; Even et al., 1985) has an Asn codon at position 31 but also harbors other coding differences. Western blot analysis of an antibody that uses V_κ R13 reveals that the other

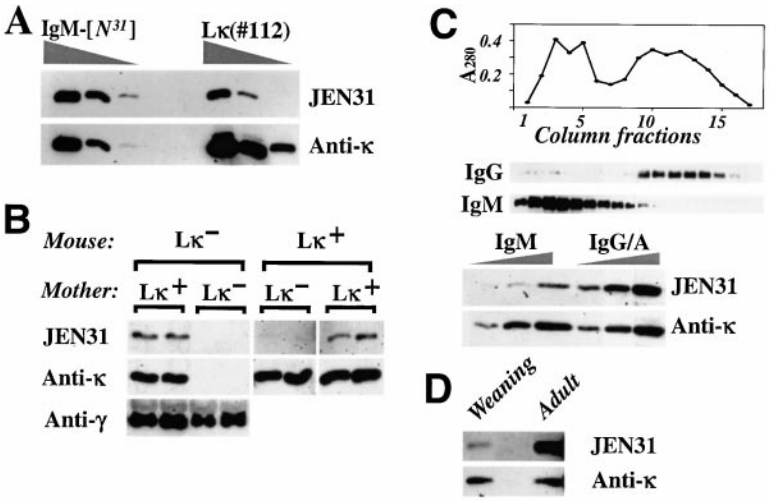

Figure 5. Monitoring the Mutation Load in Adult Mice and in Switched Isotypes

(A) The mutation load in serum immunoglobulin of 1-year-old mice. Mutation load was assessed by comparing the ratio of JEN31: anti-κ signals obtained with total serum (diluted 1:50, 1:200, and 1:800) to that obtained with pure [V_HOx1/$V_κ$Ox1-N^{31}] IgM (0.4, 0.1, and 0.025 μg). Densitometry analysis of the autoradiograph illustrated reveals that 6.5% of the Igκ chains in this serum sample react with JEN31. Similar analyses using sera from three other 1-year-old mice gave values of 5.6%, 5.0%, and 8.1%. This calculation makes the simplifying assumption that all JEN31 reactivity is due to a Ser-31→Asn mutation and that all mutated κ chains carrying Asn-31 are equally reactive with JEN31.

(B) Comparison of the levels of maternally derived and neonatally synthesized mutated antibody in young mice. Western blot comparison of JEN31-reactive material in sera from 5- to 6-week-old *Lκ*$^+$ *[Moκ*$^{-/-}$*]* or *Lκ*$^-$ *[Moκ*$^{-/-}$*]* mice born to *Lκ*$^+$ *[Moκ*$^{-/-}$*]* or *Lκ*$^-$ *[Moκ*$^{-/-}$*]* mothers. Loading was confirmed by reprobing with anti-rat κ or anti-mouse γ antisera.

(C) Comparison of the mutation load in serum IgM and IgG/A of a 1-year-old *Lκ Mo[κ*$^{-/-}$*]* mouse by JEN31 immunoblot. Serum samples were subjected to gel filtration and fractions analyzed for the presence of IgM and IgG by immunoblotting. The abundance of mutated immunoglobulin in purified IgM (15, 5, and 1.7 μl of fraction number 2) and IgG (9, 3, and 1 μl of fraction number 11) samples was determined by JEN31 immunoblot, controlling for loading using anti-rat κ. ELISA revealed that the IgM peak fraction suffered less than 1% IgG contamination. The blots reveal the mutation load in IgG/A to be 9-fold higher than in IgM; two other mice gave values of 16 and 6 for the relative IgG/A:IgM mutation load.

(D) Comparison of the mutation load in endogenously synthesized serum IgG that was purified from pooled serum samples taken from mice either at weaning or at 10 months.

coding differences prevent recognition by JEN31 [Figure 6A (i)]. Second, Moκ$^+$ mice (born to *Moκ*$^{-/-}$ mothers) have no detectable JEN31-reactive light chains at weaning but develop such JEN31 reactivity with age [Figure 6A (ii)].

Sera from germ-free mice give a much weaker JEN31 signal than do sera from age-matched conventionally housed controls (Figure 6B). This is not simply a consequence of a reduction in total serum immunoglobulin, since the ratio of JEN31:anti-κ signals is diminished by at least an order of magnitude. Thus, the mutation load in serum immunoglobulin is considerably reduced in germ-free mice.

Diminished Titers of Mutated Ig in T Cell–Deficient Mice

The ability to exploit the JEN31 mAb to monitor mutation of endogenous (as opposed to transgenic) Igκ chains opens up the possibility of ascertaining whether the accumulation of mutated serum immunoglobulin is affected in various mutant mouse strains. To improve the

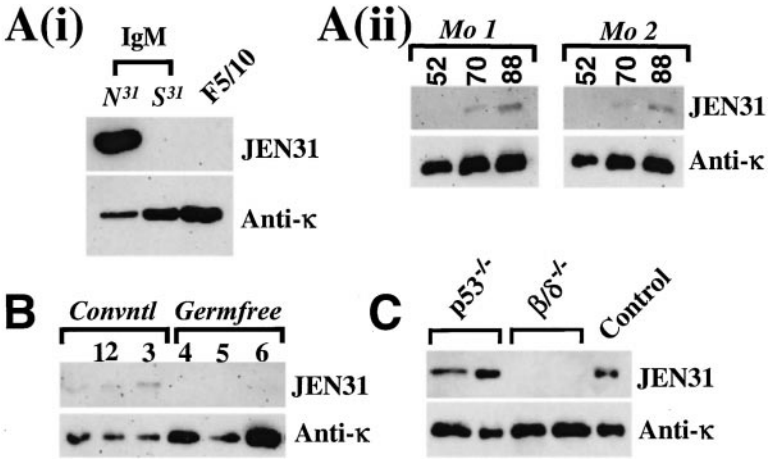

Figure 6. Mutation Accumulation Is Reduced in Germ-Free and T Cell–Deficient Mice

(A) JEN31 can be used to monitor mutation of the Igκ chains expressed by the endogenous mouse Igκ locus: (i) Western blot analysis reveals that, while JEN31 recognizes the mutated $V_κ$Ox1-N^{31} (CDR1 = SASSSVNYMH, with the N being position 31), it does not recognize the related light chain of IgG antibody F5/10 (gift from Dr G. Gilkeson; [Gilkeson et al., 1993]), which comprises an Igκ chain encoded by the unmutated $V_κ$Ox family member *R13* (κCDR1 = RASSSVNYMY). (ii) JEN31-reactive light chains develop with age in the sera of *Moκ*$^{+/-}$ BALB/c mice born to *Moκ*$^{-/-}$ mothers. The blot was controlled for loading by reprobing with anti-κ.

(B) The abundance of JEN31-reactive material was compared in sera of conventionally housed and germ-free 26-week-old SW mice. One and one-half microliters of serum from conventionally housed mice was loaded, as opposed to 15 μl of serum from germ-free mice. The germ-free mice displayed a mean serum IgM titer that was 1.5-fold greater than that of the conventionally housed animals, whereas their mean IgG and IgA titers were reduced by 6- and 13-fold, respectively.

(C) Comparison of the abundance of JEN31-reactive material in the protein A binding fraction of serum from *p53*$^{-/-}$ *TCR*β$^{-/-}$δ$^{-/-}$, and C57BL/6 (control) mice, controlling for loading using anti-κ antiserum.

sensitivity of the assay, we exploited the fact that the mutation load in IgG is higher than in IgM and performed the JEN31 immunoblot assay on the protein A binding fraction of serum immunoglobulin. As will be seen in Figure 6C, mutated endogenous Igκ chains are readily detectable in the serum of normal mice and of p53-deficient animals (which are known to mutate their immunoglobulin genes [Jolly et al., 1997]). However, the accumulation of mutated immunoglobulin is substantially diminished in the serum of *TCR*$\beta^{-/-}$*TCR*$\delta^{-/-}$ double knockouts, showing that T cells are important for the accumulation of mutated serum immunoglobulin and incidentally confirming that JEN31 has little reactivity toward the immunoglobulins present in the primary (unmutated) repertoire.

Discussion

The JEN31 monoclonal antibody has enabled us to glean information about the contribution of somatic hypermutation to the serum immunoglobulin repertoire. The bulk of the conclusions were derived by analyzing mice harboring an Igκ transgene. It is possible that a restricted primary repertoire of B cell specificities caused by imposition of this Igκ transgene might lead to increased diversification by somatic mutation. However, we do not believe that this leads us to significantly overestimate the contribution of mutation to the diversity of serum immunoglobulin in normal mice. Not only does the Igκ transgene lead to a relatively minor restriction of the primary repertoire (the mice have access to the full extent of IgH diversity), but the major conclusions are supported by experiments using JEN31 to monitor mutation of endogenous V_κOx family members in nontransgenic animals.

A straightforward conclusion to emanate from the work is that somatic mutation makes effectively no contribution to the primary repertoire of serum antibodies in the mouse. While 10-day-old mice contain abundant quantities of endogenously synthesized IgM, there is no detectable JEN31-reactive material. This does not mean that newborn mice contain no somatically mutated antibody but rather, as shown here, such antibody is exclusively of maternal origin. Thus, the selection processes involved in the shaping of the primary antibody repertoire in the mouse (unlike those in the sheep [Reynaud et al., 1995]) do not lead to the induction of hypermutation. These results extend upon (but are entirely consistent with) previous analyses of mouse V gene diversity at the cellular level. Thus, while there is no information that specifically relates to V gene diversity in the antibody-secreting cells of young mice, the V genes of splenic IgM$^+$IgD^{hi}, peritoneal CD5$^+$, and Peyers patch B cells in 4-week-old mice (which could act as precursors for some of the antibody-producing cells) are all essentially unmutated (Gu et al., 1991; González-Fernández et al., 1994).

In humans, mutated V gene sequences have been identified in samples taken from human cord blood and 10-day-old infants (Mortari et al., 1993; Ridings et al., 1997), although the mutation load is lower than that found in the tonsillar B cells of a 4-year-old (Klein et al., 1994). However, this need not mean that somatic mutation contributes to the primary repertoire in humans but, rather, that humans might be better able to mount secondary responses to foreign antigens in early life.

The JEN31 immunoblot analysis revealed that, as mice age, mutated immunoglobulin accumulates in serum even in the absence of intentional immunization. Indeed, the existence of a small fraction of B cells carrying mutated V genes has previously been identified in unimmunized mice (Schittek and Rajewsky, 1992). This mutation is probably taking place in response to environmental antigens, since mutation accumulation is greatly reduced in germ-free mice. In this respect, mutation likely parallels heavy-chain class switching: the accumulation of class-switched isotypes is greatly diminished in germ-free and antigen-free mice (Hooijkaas et al., 1984; Pereira et al., 1986).

However, this work reveals a striking difference between the kinetics of appearance of mutated and class-switched serum immunoglobulin. Most mutated immunoglobulin accumulates long after serum IgG and IgA levels have plateaued. Thus, an 8-week-old and 1-year-old mouse, while harboring similar concentrations of IgG, exhibit a two order of magnitude difference in the abundance of JEN31-reactive immunoglobulin. The intensity of the JEN31 signal essentially reflects the abundance of VκOx-containing immunoglobulin in which Ser-31 has been mutated to Asn. In heavily mutated sequences, linked CDR1 mutations could destroy JEN31 reactivity, leading to a slight underestimation of the abundance of mutated sequences, although this effect is likely to be minor. The increase in JEN31 reactivity will reflect increases both in the proportion of serum antibodies that are mutated as well as in the extent of mutation within the mutated population. It is an increase in the proportion of serum antibodies that are mutated that must account for a major component of the two log increase in JEN31 signal that accompanies aging. Thus, while mutation does not contribute to the primary repertoire, somatically mutated antibody accounts for a major proportion of serum immunoglobulin in a 1-year-old mouse. Given the importance of "natural" (preimmune) antibody in facilitating immune responses (Boes et al., 1998; Ehrenstein et al., 1998), the change in mutation load (and therefore serum immunoglobulin repertoire) that accompanies aging could well be part of the cause of the age dependence of responsiveness to administered antigens.

The rise in mutation load with age presumably reflects that an increasing proportion of the serum antibody derives from memory B cells generated during previous encounters with antigen (for example, see Smith et al., 1997). This tendency to increased mutation load will be particularly enhanced if memory B cells can undergo further mutation on repeated rounds of antigen encounter—an issue that has excited some discussion (Siekevitz et al., 1987; Rada et al., 1991; González-Fernández et al., 1994). However, a paucity of mutation in the IgG fraction of young mice would also result if much of the class switching in young animals occurred without concomitant hypermutation. Indeed, the two processes are not inextricably linked. Thus, although both switching and mutation can take place in germinal center B cells, extrafollicular B cells are able to differentiate into antibody-secreting cells following class switching without accompanying hypermutation (Jacob and Kelsoe, 1992; Toellner et al., 1996). Furthermore, in vitro studies indicate that mutation and switching can be induced by different signals (see, for example, Manser, 1987; Razanajaona et al., 1997; Zan et al., 1999).

The fact that the increasing mutation load in serum

IgG is accompanied by little change in total IgG concentration also implies a significant turnover in the serum antibody repertoire. Thus, if plasma cells maintain antibody secretion without undergoing further hypermutation, then the long-lived plasma cells that have been recently described (Manz et al., 1997; Slifka et al.,1998) can only account for a limited proportion of the total serum antibody repertoire.

The mutation load in adults is not evenly distributed between serum IgM and IgG; switched isotypes carry a 10-fold greater mutation load. Thus, the repertoire of serum IgM specificities remains substantially biased toward the primary (antigen-independent) repertoire—a result consistent with immunoreactivity profiling (Hooijkaas et al., 1985; Bos et al., 1989; Haury et al., 1997). In contrast, the major proportion of immunoglobulins of switched isotypes in adults are mutated. Switched isotypes account for the majority of serum immunoglobulin (though not the majority of B cells) in adults; therefore, at least in respect of its effector arm (if not at the level of B cells), the humoral immune system resembles the cellular system (reviewed in Hodes, 1997) in displaying a major overall shift from primary to secondary repertoire with age.

While we have predominantly used the JEN31 mAb to monitor mutation in serum antibodies that comprise a transgenic κ light chain, the same JEN31 antibody can be used with somewhat reduced sensitivity to monitor mutation of endogenous immunoglobulins in normal mice, presumably by recognizing mutated versions of endogenous VκOx1 and related Vκ segments. Such assays reveal that the accumulation of mutated immunoglobulin is substantially diminished in T cell–deficient animals. The results, however, do not allow us to discriminate whether this diminution in mutation reflects a failure to induce mutation or a failure to select and expand B cells expressing mutated immunoglobulin. Nevertheless, it should be possible to develop the assay into a rapid screen of antibody hypermutation in mice generated in both gene targeting and random genome mutagenesis projects.

Experimental Procedures

Mice

All sera were obtained from mice bred and maintained in our barriered, specific pathogen free facility except for those from 26-week-old germ-free (and control) SW mice, which were obtained from Taconic (Germantown, NY), and *TCR*$\beta^{-/-}\delta^{-/-}$ mice, which were generously provided by Dr. Mike Owen (ICRF, London). Screening for pathogens at autopsy was performed by Harlan UK according to their M1 screen. Mice carrying the Lκ and LκΔ[Int$_{J\text{-}C}$, 3′Fl] transgenes have been previously described (Sharpe et al., 1991; Klix et al., 1998). Mice carrying a targeted disruption of their endogenous Igκ locus (*Mo*κ$^{-/-}$ mice, bred on a BALB/c background; [Zou et al., 1995]) were a gift from Marianne Brüggemann (Cambridge), and μ*MT* mice (Kitamura and Rajewsky, 1992) were obtained from Werner Müller and Klaus Rajewsky.

Isolation of JEN31 mAb

Eight mice were challenged i.p. with CJ1 (NH_2-CSASSSVNYMHWY COOH; corresponding to the CDR1 of N^{31}-V_κOx1) that had been coupled to thyroglobulin using maleimidobenzoyl-N-hydroxysuccinimide (Pierce, Chester, UK). After three i.p. boosts (100 μg) at 6- to 8-week intervals and a final i.v. immunization with 50 μg CJ1-thyroglobulin, spleens were removed and fusion performed with the NS0 plasmacytoma. Supernatants were screened for CJ1-specific antibody by ELISA on plates coated with CJ1-BSA and then secondary screens performed on [V_HOx1/V_κOx1-S^{31}] IgM and [V_HOx1/V_κOx1-N^{31}] IgM. The JEN31 mAb was derived from a fusion performed using the spleen from a hyperimmunized (C57BL/6xCBA/Ca)F1 mouse.

Recombinant Antibodies and Fabs

Plasmid #1299, directing expression of the μ heavy chain of the recombinant V_HOx1/V_κOx1-N^{31} and V_HOx1/V_κOx1-S^{31} 2-phenyloxazolone-specific IgMs, is based on pSV-Vμ1 (Neuberger, 1983). The NP-specific V_H region of pSV-Vμ1 (between the PstI and BstEII sites) in #1299 was replaced by the equivalent region of V_HOx1 (generated by RT-PCR from hybridoma mRNA). The Lκ transgene in pSV2neo (Sharpe et al., 1991) was used to direct expression of [V_κOx1-S^{31}]/ratCκ Igκ light chains. The plasmid directing expression of the [V_κOx1-N^{31}]/ratCκ Igκ light chains is similar, except that the region between the KpnI and PstI sites in the V_κOx1 CDR1 have been replaced by a synthetic oligonucleotide yielding an AGT→AAT substitution at codon 31. The μ heavy chain–expressing plasmid was transfected together with one of the Igκ-expressing plasmids into plasmacytoma NS0. Recombinant IgM was purified from the supernatants of cloned transfectants by affinity chromatography on 2-phenyloxazolone-conjugated Sepharose.

Vectors for recombinant Fab expression are analogous to the human IgG1,κ Fab vector described by Griffiths et al. (1994) but with the V genes provided by V_HM21/J_H2 and V_κOx1/J_κ5 (or an engineered Ser-31→Asn mutant of V_κOx1) that were PCR amplified from hybridoma NQ22/61 (Berek et al., 1987). The recombinant κ chain carried a c-myc epitope and His_6 tag at its C terminus (Low et al., 1996), allowing purification on Ni-NTA columns (Qiagen) from the periplasm of *E. coli* TG1 transformants that had been induced with IPTG.

Fractionation of Serum

For separation of IgM and IgG/A, mouse serum was subjected to gel filtration on Sephacryl S300 and the IgM-, IgG-, and IgA-containing fractions identified by both class-specific ELISA and Western blotting. The protein A binding fraction of serum immunoglobulin was purified by mixing serum (100–200 μl) with protein A-Sepharose (25 μl packed volume) and eluting by boiling in SDS/PAGE sample buffer after washing.

ELISAs and Western Blotting

For Western blotting with JEN31, samples were subjected to SDS/PAGE on 12% gels, transferred onto PVDF membranes (Millipore), and membranes blocked with PBS/5% Marvel. Membranes were incubated with biotinylated JEN31 (generated by conjugating NHS-biotin [Pierce] to JEN31 that had been purified on protein A-Sepharose from hybridoma supernatants) in PBS/5% Marvel and then developed with streptavidin/horseradish peroxidase (Pierce or Binding Site, Birmingham, UK; diluted according to manufacturer's instructions) prior to visualization by ECL (Amersham, High Wycomb, Berks, UK). Biotinylated probe was subsequently washed off the filters by incubating in 60 mM Tris-HCl (pH 6.8), 2% SDS, and 100 mM 2-mercaptoethanol at 65°C for 30 min followed by PBS/0.05% Tween 20 for 15 min. After checking loss of signal, filters were reprobed with sheep anti-rat κ antiserum followed by horseradish peroxidase–conjugated donkey anti-sheep serum (both from Binding Site) and ECL. The relative specific mutation load in different samples was assessed by comparing the amounts of total immunoglobulin from those samples (assaying using anti-κ) that were needed to achieve an equivalent JEN31 signal when the JEN31 signal was within the range of assay proportionality.

Titration of serum levels of immunoglobulin containing transgenic κ chains was performed by a sandwich ELISA using MRC-OX12 mouse anti-rat κ mAb as previously described (Sharpe et al., 1991). Measuring the abundance of serum IgM, G, and A that contained transgenic κ chains was performed by diluting the serum samples in PBS/1%-normal rabbit serum/1%-*[Mo*κ$^{-/-}$*]* serum, assaying the dilutions by ELISA on plates coated with rabbit isotype-specific antisera (anti-IgM and anti-IgG from Jackson, PA; anti-IgA from Zymed, CA), and developing with biotinylated sheep anti-rat κ and HRP-conjugated donkey anti-sheep serum.

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